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First record of the bacterial endosymbiont *Wolbachia* for phytophagous hoverflies from genus *Merodon* (Diptera, Syrphidae)

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Abstract. *Wolbachia* is a widespread bacterial endosymbiont among arthropod species. It influences the reproduction of the host species and also mtDNA diversity. Until now there were only a few studies which detected *Wolbachia* infections in hoverflies (Diptera, Syrphidae), and this is the first broader study with the aim to examine the incidence of *Wolbachia* in the hoverfly genus *Merodon*. The obtained results indicate an infection rate of 96% and the presence of both *Wolbachia* supergroup A and B, which are characteristic for most of the infected arthropod species. Additionally, the presence of multiple *Wolbachia* strains in the *M. aureus* group species was detected and the mtDNA *COI* based relationships of the group were discussed in the light of infection. Finally, we discuss plant mediated horizontal transmission of *Wolbachia* strains among the studied hoverfly species.

Keywords: 16S rRNA gene, *Drimia maritima, Merodon aureus* group, *wsp*.

INTRODUCTION

The hoverflies (Diptera, Syrphidae) are a widely distributed insect family present almost everywhere in the world except on the Antarctic and remote oceanic islands (Thompson & Rotheray, 1988). The hoverfly genus *Merodon* Meigen, 1903 (subfamily Eristalinae) is distributed over Palaearctic and Palaeotropical regions (Hurkmans 1993). The immature stages of *Merodon* species develop in and feed on underground storage organs of geophytes of the families Asparagaceae, Iridaceae and Amaryllidaceae (Andrić *et al.* 2014; Ricarte *et al.* 2008, 2017; Preradović *et al.* 2018). Adults morphologically mimic hymenopterans and feed on pollen and nectar (Hurkmans 1993). The genus comprises more than 160 species (Ståhls *et al.* 2009; Vujić *et al.* 2012), however, the real number of *Merodon* species is still unknown considering high level of diversity and detected presence of cryptic species (e.g. Milankov *et al.* 2008, 2009; Radenković *et al.* 2011; Vujić *et al.* 2012; Popović *et al.* 2015; Ačanski *et al.* 2016; Šašić *et al.* 2016, 2018; Veselić *et al.* 2017).

The *Merodon aureus* hoverfly species group is taxonomically especially challenging, as it comprises a high genetic diversity with minor or lacking differences in morphological traits (Šašić *et al.* 2016). The group comprises species morphologically close to *M. aureus* Fabricius, 1805. The members of the taxa are small sized (8-13 mm), with a short, rounded abdomen, a distinct spike on the metatrochanter in males and a characteristic structure of the male genitalia (Vujić *et al.* 2007; Radenković *et al.* 2011). Until recently, the group comprised altogether 18 previously-known and newly-discovered taxa from the Mediterranean region and southern European mountain regions (Marcos-García *et al.* 2007; Vujić *et al.* 2007; Milankov *et al.* 2008; Radenković *et al.* 2011; Speight, 2014), while new data indicates the presence of additional species (Šašić *et al.* 2016, 2018; Veselić *et al.* 2017; Radenković *et al.* 2018).

The first results about molecular diversity of *Merodon aureus* group were based on analyses of 3' and 5' fragments of the mtDNA *COI* gene and suggested the presence of multiple cryptic species complexes within the group. The morphological character states usually used in taxonomy of hoverflies mostly failed to discern these potential species (Šašić *et al.* 2016; Radenković *et al.* 2017). However, subtle differences in wing and surstylus shape were detected using geometric morphometry (see Ačanski *et al.* 2016; Šašić *et al.* 2016; Radenković *et al.* 2017). Maximum likelihood (ML) analysis of *COI* sequences including representatives of all potential complexes from *M. aureus* species group revealed deep divergences between morphologically close species (Fig. 1). The obtained molecular evidence showed clear conflict with the morphologically defined subgroups and/or species complexes (Vujić, personal communication; Šašić *et al.* 2016; Radenković *et al.* 2017).

Figure 1.

Over the last few decades, mtDNA has been the most popular marker for quantifying molecular diversity, as the marker contains a combination of technical benefits (ease of amplification), and supposed biological and evolutionary advantages such as clonality, near-neutrality and often clocklike nature of its substitution rate. However, mtDNA is not always clonal, not neutrally evolving and not clocklike, which brings into question its use in recovering recent species and population histories (e.g. Galtier *et al.* 2009). In addition to these limitations of use, the taxonomic utility of the maternally inherited mitochondrial genome could be compromised by the presence of symbiotic bacteria, which pass from a female to its offspring (Galtier *et al.* 2009). The most important of the so-called "reproductive parasites" is *Wolbachia pipientis* (Alphaproteobacteria: Rickettsiales: Rickettsiaceae), which is facultative endosymbiont estimated to have infected more than half of arthropod species (Weinert *et al.* 2015). Although other bacterial reproductive parasites are also known (*Cardinium*, *Arsenophonus*, *Rickettsia*, *Spiroplasma*), *Wolbachia* is the most abundant

endosymbiont among arthropod species and with broadest range of host reproductive phenotypes including induction of cytoplasmic incompatibility, feminisation of genetic males, parthenogenesis and male killing (Duron *et al.* 2008; Zug & Hammerstein, 2012).

According to molecular phylogenetic analyses *Wolbachia pipientis* has been divided into seventeen clades (A-R, except G which is recombinant of A and B supergroups), termed supergroups (Werren *et al.* 1995; Bandi *et al.* 1998; Vandekerckhove *et al.* 1999; Lo *et al.* 2002, 2007; Czarnetzki & Tebbe 2004; Baldo & Werren, 2007; Bordenstein *et al.* 2009; Haegeman *et al.* 2009; Ros *et al.* 2009; Augustinos *et al.* 2011; Bing *et al.* 2014; Glowska *et al.* 2015; Wang *et al.* 2016). The supergroups taxonomic status was discussed in Ramírez-Puebla *et al.* (2015) who suggested that *Wolbachia* supergroups represent separate evolutionary lineages and that they should be designated as species. They also indicated that some of the supergroups could contain more than one *Wolbachia* species. The proposed nomenclature is criticized by Lindsey *et al.* (2016) as inadequate and confusing.

Wolbachia is probably the most widespread endosymbiont of arthropods and nematodes (Charlat *et al.* 2003; Werren *et al.* 2008). Recent studies estimated 19% to 76% infection rates of *Wolbachia* among arthropod species (Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000; Hilgenboecker *et al.* 2008; Werren *et al.* 2008; Simões *et al.* 2011; Weinert *et al.* 2015; Zug & Hammerstein, 2012). The evolutionary success is achieved through a variety of effects on host biology, ranging from manipulation of reproduction in favor of females to mutualistic interactions with host species. *Wolbachia* interact with host sex-determination systems and the cell cycle, and its effect on host populations can frame sexual behaviors and species diversity (Charlat *et al.* 2003). The infection is maternally inherited via infection of developing oocytes or it can be a consequence of horizontal transmission (Werren, 1997). It is most likely to find *Wolbachia* in ovaries, although it can also occur at high intensities in the

fat body and other tissues (e. g. Werren, 1997; Dobson *et al.* 1999; Albertson *et al.* 2009; Pietri *et al.* 2016).

The first test for the presence of *Wolbachia* in hoverfly species (Syrphidae) was the study of Werren and Windsor (2000) who found that the Nearctic species *Milesia virginiensis* tested negative for the presence of *ftsZ* bacterial cell-cycle gene of *Wolbachia*. In 2006, Sintupachee *et al.* found *Syritta rufifacies* negative for the *ftsZ*, but the species *Graptomyza brevirostris* (Eristalinae: Volucellini) tested positive for both *ftsZ* and a *Wolbachia* surface protein (*wsp*) genes. Evison *et al.* (2012) screened pollinator groups in the UK for different groups of parasites including *Wolbachia*, and among the tested species they included four species of hoverflies, *Rhingia campestris* (Eristalinae: Rhingiini), *Eristalis arbustorum* and *E. tenax* (Eristalinae: Eristalini) and *Episyrphus balteatus* (Syrphinae: Syrphini), which all were positive for the tested *CoxA* primers. However, the current molecular taxonomy of hoverflies and applying mitochondrial markers neglect the potential bias of *Wolbachia* on the results and do not contain any *Wolbachia* screening test.

In this study, we estimate the incidence of *Wolbachia* in the genus *Merodon* (Diptera, Syrphidae) using samples from recent field collections across South European countries, Austria, Romania, Turkey, Iran, Morocco and the South African Republic. We amplified and sequenced bacterial marker genes (16S rRNA gene and *wsp* gene) with the aim to assign *Wolbachia* supergroups present in *Merodon* hoverflies. We particularly focus on the screening of *M. aureus* group species in the light of the observed high mtDNA *COI* gene variability, which is incongruent with morphological invariability in several species complexes, and test for coevolution between *M. aureus* group and *Wolbachia* strains infecting the species of the group. Additionally, we performed screening of host plant bulb with the aim to prove the presence of *Wolbachia* in plant tissue and discuss potential horizontal transmission via bulb.

MATERIAL AND METHODS

Specimens analyzed

The hoverfly specimens were collected from 2012 to 2016 and identified to the species, or subgroup (*Merodon aureus* group specimens) level (by Dr Ante Vujić and according to Šašić *et al.* 2016; Radenković *et al.* 2017). The bulb of *Drimia maritima* (syn. *Urginea maritima*) which is a host plant of *M. luteihumerus* larvae was collected in March 2017. All the data about collected samples are provided in Table S1.

DNA extraction

DNA extractions of 2 - 3 legs and separately of abdomens of the hoverfly specimens was performed by using the SDS extraction protocol according to Chen *et al.* (2010). The gDNA extracted from legs were used for *Wolbachia* specific 16SrRNA gene amplification, while the gDNA extracted from abdomens were used for *Wolbachia wsp* gene amplification. The main reason for repeated gDNA extraction was low amplification success of *wsp* gene using gDNA extracted from legs, which is probably a consequence of lower amount of bacterial DNA in legs comparing to the abdomen (as previously mentioned, the highest concentration of *Wolbachia* is in reproductive tissue).

Testing for the presence of *Wolbachia*

16S rRNA gene amplification

Primary screening on *Wolbachia* presence was based on the amplification of *Wolbachia*'s 16S rRNA gene fragment. In total, 74 specimens belonging to different *Merodon* species

were screened for *Wolbachia* presence based on amplification and sequencing of bacterial 16S rRNA gene. 45 of these belong to *M. aureus* group, with fewer samples from the following species groups: five from *M. avidus* group, five from *M. nanus* group, three from *M. geniculatus* group, three from *M. albifrons* group, three from *M. constans* group, three from *M. natans* group, three from *M. nigritarsis*, three from *M. desuturinus* group, and one from the species *M. luteihumerus*. We tested 1-3 specimens per species (Table 1).

16S rRNA gene fragment was amplified using WspecF and WspecR primer pair (Werren & Windsor, 2000). Polymerase chain reactions (PCR) were carried out in 25 µl reaction volumes. The reaction mixture contained 1x Taq Buffer without $MgCl_2$ (ThermoScientific, Lithuania), 1.5 mM $MgCl_2$, 0.25 mM of each nucleotide, 1.25 U Taq polymerase (ThermoScientific, Lithuania), 7 pmol of each primer, and approximately 50-100 ng template DNA. The amplification of the bacterial 16S rRNA gene was carried out following the protocol described in Werren & Windsor (2000).

The PCR products were checked on 1.5% agarose gels and the PCR product from gDNA of *Drosophila melanogaster* extracted from line 5 from Bloomington stock center http://flystocks.bio.indiana.edu/Reports/5.html (project number: OI 173012) was used as a positive control. Additionally, we also used PCR reaction mixture without gDNA as negative control in order to eliminate potential contamination.

Wsp gene amplification

In addition to 16S rRNA gene, we tested *Merodon aureus* group specimens on bacterial *wsp* gene. For this purpose, we extracted additional genomic DNA from the abdomen of hoverflies (see above). We used Phire Animal Tissue Direct PCR Master Mix (ThermoScientific, Lithuania) to amplify *wsp* gene according to the manufacturer's

instructions. The same kit was used for direct *wsp* gene amplification from the tissue of *Drimia maritima* bulb and the larvae of *M. luteihumerus* discovered within the bulb. The primers used to amplify the *wsp* fragment are 136F, 691R, 81F, 522R (Zhou *et al.* 1998). The PCR was performed with three primer pair combinations: 136F/691R for *Wolbachia* supergroup A, 81F/ 522R for *Wolbachia* supergroup B, and 81F/691R for both supergroups (Zhou *et al.* 1998). Initially, we screened all samples with *wsp* primer combination for supergroup A. The samples without products were additionally tested with *wsp* primer combination for supergroup B or universal combination for both supergroups. Only amplification products with a single bend on 1.5% agarose gels were used for sequencing.

Sequencing

The PCR products are enzymatically purified using exonuclease I and shrimp alkaline phosphatase enzymes. Sequencing was done in both directions using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, Ca, USA) at the Sequencing Service Laboratory of the Finnish Institute for Molecular Medicine (FIMM), Helsinki, Finland.

16S rRNA gene and wsp gene sequences analyses

The produced 16S rRNA gene and *wsp* gene sequences were blasted against the nucleotide collection database at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) using Megablast optimized for highly similar sequences. *Wsp* sequences were also checked against the *Wolbachia* MLST database (Baldo *et al.* 2006). Finally, the screening results were presented in a form of a table with marked specimens where *Wolbachia* was identified using 16S rRNA gene, *wsp* or both genes (Table 1). All

sequences have been submitted in GenBank under accession numbers MK184213 – MK184277 (16S rRNA gene) and MK192943 – MK192981 (*wsp*), while *wsp* sequences are additionally deposited in MLST database (Table 2, Table S1).

In order to place *Wolbachia* detected in *Merodon* specimens in a particular supergroup, we constructed ML tree based on 16S rRNA gene sequences. The sequences were manually aligned and the tree was constructed using RAxML 8.2.8 (Stamatakis, 2014) through the CIPRES Science Gateway web portal (Miller *et al.* 2010) and by applying the general time-reversible (GTR) evolutionary model with gamma distribution (Rodriguez *et al.* 1990), while the statistical support for the clades was assessed using the rapid bootstrap method with 1000 replicates. The analysis also included 29 sequences belonging to *Wolbachia* supergroups (A, B, F, H, I, M, N, O) founded in insect hosts, which were downloaded from GenBank (see Table S2). As outgroups, we used two species of α-Proteobacteria: *Ehrlichia canis* and *Anaplasma marginale* (GenBank accession numbers: M73226, M60313), and the tree was rooted on *Anaplasma marginale.* In order to test cophylogeny between *M. aureus* group *COI* tree and *Wolbachia* 16S rRNA gene tree, we applied Procrustean Approach to Cophylogeny (PACo) in R environment (R Core Team, 2018) as described in Balbuena *et al.* (2013). As input data, we used unrooted ML trees. For 16S rRNA gene we firstly determined sequence types by using DnaSP 5 software (Librado & Rozas, 2009) which was used for an unrooted ML tree construction in RAxML 8.2.8 (Stamatakis, 2014).

For *wsp* sequences, alignment was performed using the L-INS-I strategy as implemented in MAFFT (Katoh & Standley, 2013) available on the EMBL-EBI bioinformatics framework (McWilliam *et al.* 2013). The total number of alleles was determined by using DnaSP 5 software (Librado & Rozas, 2009). The assessments of pairwise differences, uncorrected p distance values between alleles, were conducted in MEGA7 (Kumar *et al.* 2016). This gene is not used for phylogeny reconstruction as the evolutionary signal is masked by its mosaic

nature, however, it can be used for strain typing based on a combination of four hypervariable regions (HVRs) (Baldo *et al.* 2005, 2006). Thus, for each *wsp* allele, we determined HVRs profile by checking against the *Wolbachia* MLST database (Baldo *et al.* 2006).

RESULTS

The primary screening on *Wolbachia* using 16S rRNA gene as a marker was performed on 52 species of the genus *Merodon* or 74 specimens from which ten specimens and five species tested negative. Within the *M. aureus* group, three specimens belonging to *M. sapphous* sp. n. 2, *M. aureus* sp. n. 2 and *M. balkanicus* tested negative, within *M. segetum* (*M. natans* group) as well as within *M. melanocerus* (*M. desuturinus* group) two specimens tested positive, while one was negative. *M. albifrons* (*M. albifrons* group) specimens were all negative, while in *M. nanus* (*M. nanus* group) one specimen was positive and one was negative for *Wolbachia* infection. The one tested *M. luteihumerus* specimen were also negative. The screening results are summarized in Table 1.

Table 1.

For ML tree construction we used 16S rRNA gene sequences. The aligned sequence set used in the analysis was 415bp long. All *Wolbachia* 16S rRNA gene sequences produced in this study are resolved as supergroup A, except the *Wolbachia* sequences from *M. neofasciatus* which are resolved with supergroup B sequences (Fig. 2).

Figure 2.

PACo analysis resulted in a residual sum of squares $m^2xy = 0.355$, under the probability value P=0.064. Thus, the cophylogeny hypothesis between *M. aureus* group *COI* tree and *Wolbachia* 16S rRNA gene tree was rejected as statistically insignificant (P>0.05). The

relationships between *COI* sequences of *M. aureus* group specimens and corresponding *Wolbachia* endosymbionts (based on 16S rRNA gene sequences) are presented in Figure 3.

Figure 3.

In order to achieve better resolution in *Wolbachia* strain determination, we additionally tested *Merodon aureus* group specimens (41 species or 45 specimens) for the *wsp* gene product. The amplification products were detected in 38 species (42 specimens). *M. nisi*, *M. unicolor* and *M. balkanicus* tested negative. Multiple products of *wsp* amplification were detected in *M. naxius*, *M. andriotes* and *M. puniceus*, and these amplification products were not further processed. In total 39 sequences which correspond to *M. aureus* group specimens were produced, however, four of them were discarded because of poor quality. The *wsp* gene was also amplified and sequenced for *Drimia maritima* bulb, and *M. luteihumerus* larvae (3 specimens) from the host plant bulb (Table 1).

The final *wsp* sequence matrix contained 39 sequences. The aligned sequences were 560bp long, and with gap regions (see Fig. S1). We discovered 7 different *wsp* alleles, from which the A1 was most common among *Merodon aureus* group specimens, but present also in the bulb and *M. luteihumerus* larvae (see Fig. 2). Based on Blast search results the sequences were 99% to 100% identical to previously discovered *Wolbachia* strains *wsp* sequences from different insect hosts, except A6 which is 97% identical to *wsp* sequence from *Ceutorhynchus obstrictus* (cabbage seed pod weevil) (see Table S3). A1 is identical to *Wolbachia wsp* sequences from *Formica sanguinea, Formica exsecta* (both ants), *Protocalliphora sialia* (birdnest blowfly), *Conotrachelus nenuphar* (plum curculio), and *Ceutorhynchus obstrictus* (cabbage seed pod weevil). A4 is identical to *Ectemnius continuus* (a wasp species) *wsp* sequence (although the query cover is 96%).

By checking the *wsp* sequences against *Wolbachia* MLST database, we found that A1 sequence is identical to *wsp* allele 311, while rest of the alleles are detected for the first time in this study and they are submitted in the database as new alleles. Additionally, the DNA sequences of all alleles were translated and HVR peptides are determined. The new HVR peptides are submitted in the aforementioned database. The WSP profiles for each of alleles are presented in Table 2.

Table 2.

The number of base differences per site between alleles (uncorrected p distances) is shown in Table 3. The analysis involved 7 nucleotide sequences of *wsp* alleles. All ambiguous positions were removed for each sequence pair. The smallest p distance has been detected between A1 and A2, while the most divergent are A3 and A7.

Table 3.

The *Wolbachia* detection success was similar when comparing PCR amplification between the two applied molecular markers (16S rRNA gene and *wsp* gene) on *Merodon aureus* group specimens for which both markers were used. In both cases 42 out of 45 analyzed specimens had amplification product, in one there was no product (*M. balkanicus* specimen), while in four we got amplification product for only one of the markers. When comparing sequence quality, 10% of *wsp* sequences had low quality and could not be used for further analysis, while all of the 16S rRNA gene sequences were good quality sequences. Low sequence quality could be due to multiple infections by different bacterial strains, but also could be caused by contamination.

DISCUSSION

The presented results indicated a markedly high incidence of *Wolbachia* infection in *Merodon* hoverflies confirmed either by one or both amplified and sequenced *Wolbachia* genes, 16S rRNA or *wsp*. In total 50 out of 52 analyzed *Merodon* species were positive for *Wolbachia* giving an infection rate of 96%. According to the estimation of Jeyaprakash and Hoy (2000), the infection rate in arthropods reaches up to 76% (48 arthropod species out of 63 tested positive) indicating a wide distribution of *Wolbachia* infection. However, it is important to point out that in this research the estimation of infection rate is based on less than third known *Merodon* species and probably deviated from the real infection rate.

Despite wide *Wolbachia* distribution among arthropod species, the study of Bailly-Bechet *et al.* (2017) conducted on 1100 species showed that most of the species acquired *Wolbachia* only recently and the most acquisition/loss events of *Wolbachia* occurred within the last million years. These events are most likely due to imperfect maternal transmission, although in some extant because of *Wolbachia* extinction from the population. However, there are some cases which indicate longterm *Wolbachia* infection. Taking into account population level events, Bailly-Bechet *et al.* (2017) estimated that mitochondria typically accumulate 4.7% substitutions per site during an infected episode, and 7.1% substitutions per site during the uninfected phase, which means that uninfected lineages acquire *Wolbachia* every 9.3 million years, while infected lineages lose their infection every 7 million years. Assuming this scenario, it is possible that *Wolbachia* acquisition/loss dynamic shapes mtDNA genealogy of the species.

In the case of *Merodon aureus* group a potential explanation for morphologically close species splitting into two main clades on *COI* tree could be a consequence of *Wolbachia*

influence on early evolution of different mtDNA lineages within the group. This means that ancient *Wolbachia* infection shaped *COI* based phylogeny of the group. However, apparently, there is no obvious pattern of coevolution of *Wolbachia* and *M. aureus* group species when comparing *Wolbachia* 16S rRNA gene tree and *COI* gene tree of hosts.

The species within complexes of *Merodon aureus* group often shared *wsp* alleles which indicates infection by the same strain. In these cases, *Wolbachia* could influence speciation if the same strain invaded different populations independently and by coupling and spreading different mtDNA haplotypes in populations. There is evidence that *wsp* detected *Wolbachia* strains could have different variants as consequence of deeper molecular variability associated with transposable elements, as found in detailed studies of *Wolbachia* variation in *Drosophila*, *Culex*, and *Hypolimnas bolina* (Duron *et al.* 2005; 2006; Riegler *et al.* 2005; Charlat *et al.* 2009). These small differences can affect the choice of mtDNA haplotypes which will be spread together with particular *Wolbachia* strain (Charlat *et al.* 2009). The theoretical modelling (Telschow *et al.* 2007) and experimental studies on many organisms (Bordenstein *et al.* 2001; Jaenike *et al.* 2006; Koukou *et al.* 2006; Miller *et al.* 2010), including both interspecific and intersemispecific analyses, show that *Wolbachia* can promote speciation in their hosts by inducing reproductive isolation, through development of either post- and/or premating mechanisms. However, more specimens per species should be tested for *Wolbachia* before any final conclusion about the influence of infection on speciation in complexes of *M. aureus* group. Additionally, cases of potential multiple infections by different *Wolbachia* strains (indicated in Table 1 as cases where sequence quality was low * or multiple bands on electrophoresis gels were detected **) deserve an in-depth study beyond the present study.

The sequencing of only one or two *Wolbachia* genes is unlikely to reveal much about *Wolbachia* transmission between host species (Stahlhut *et al.* 2012). The characteristic transmission dynamics and cases of multiple infections of the same host with different *Wolbachia* strains have resulted in a freely recombining intracellular bacterial community and mosaic bacterial genome structure (Klasson *et al.* 2009). The comparisons of shared polymorphisms between *Wolbachia* strains confirm a mosaic structure of the *wsp* gene, which is particularly prone to recombination and is under directional selection (Schulenburg *et al.* 2000; Jiggins *et al.* 2001; Baldo *et al.* 2005). The frequent recombination events produce a high level of sequence variability which makes *wsp* an excellent single marker for distinguishing among different *Wolbachia* strains (Stahlhut *et al.* 2010). On the other hand, frequent recombination disables tracing strain genealogy and makes *wsp* a bad choice for studying *Wolbachia* horizontal transmission (Stahlhut *et al.* 2012). However, we found the same *wsp* alleles present in both the host plant *Drimia maritima* bulb tissue and in the larval specimens of *Merodon luteihumerus* acquired from the plant bulb. Although the presence of *Wolbachia* DNA is not confirmation of the presence of living bacteria (see also Kolasa *et al.* 2017), these findings reveal a potential way of horizontal transmission mediated by plants. All known early stages of *Merodon* species are found in underground storage organs of geophytes (Ricarte *et al.* 2017; Preradović *et al.* 2018).

Plant mediated horizontal transmission of *Wolbachia* has already been hypothesized by Sintupachee *et al.* (2006). They showed that four taxonomically diverse insects feeding on the same host plant contained very closely related *Wolbachia* strains, suggesting the potential role of host plants in *Wolbachia* horizontal transmission. Yang *et al.* (2013) also showed that identical strains of *Wolbachia* are shared by two species, the gall wasp *Andricus mukaigawae* and its inquiline wasp *Synergus japonicas*, which larvae feed on modified plant tissue of the gall. Ahmed *et al.* (2016) found evidence for several new instances of *Wolbachia* horizontal

transmissions in Lepidoptera, and their findings suggested that specific shared food sources and shared natural enemies were possible routes of horizontal transmission. The DNA of two different *Wolbachia* strains including the one present in asparagus beetles, *Crioceris quinquepunctata* and *C. quatuordecimpunctata*, are also detected in host plant (*Asparagus*) tissues (Kolasa *et al.* 2017) once again indicating the possible route of horizontal transmission mediated by plants. This is strongly confirmed in the study by Li *et al.* (2017) where *Wolbachia* was visualized in plant tissue, both in the phloem vessels and in some spherules along the phloem. At present, however, neither the mechanisms nor processes of *Wolbachia* horizontal transmission are completely understood. For providing more solid support for the role of the host plants in *Wolbachia* transmissions in *Merodon* hoverflies, systematic screening for *Wolbachia* should be undertaken and include both the adult flies and their developmental stages, as well as the host plants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Figure S1. The sequence alignment of *wsp* alleles.

Table S1. Data on specimens screened for *Wolbachia* infection.

Table S2. The list of *Wolbachia* 16S rRNA gene sequences representing bacterial supergroups present in insect hosts.

Table S3. Blast search results for *Wolbachia* endosymbionts of *Merodon* species and *Drimia maritima* bulb *wsp* gene sequences.

670 Figure legends and table captions

671 Figure 1. Maximum likelihood tree of *Merodon aureus* group based on combined 3' and 5'
672 *COI* sequences (Šašić *et al.* 2016; Radenković *et al.* 2017; Šašić *et al.* unpublished data).
673 Bootstrap values ≥50 are presented near nodes.

674 Figure 2. Maximum likelihood tree based on 16S rRNA gene sequences of *Wolbachia* strains
675 present in genus *Merodon*. Bootstrap values ≥50 are presented near nodes. The specimens are
676 marked with DNA IDs and the host species names or GenBank accession numbers.

677 Figure 3. Phylogeny comparison between *COI* Maximum likelihood tree of *Merodon aureus*
678 group (left) and 16S rRNA gene Maximum likelihood tree of corresponding *Wolbachia*
679 endosymbionts (right). Bootstrap values ≥50 are presented near nodes.

680 Table 1. List of specimens tested on *Wolbachia* infection.

681 Table 2. The list of *wsp* alleles from *Merodon aureus* group host species including *Drimia*
682 *maritima* bulb and *Merodon luteihumerus*.

683 Table 3. Uncorrected p distance values (%) between *wsp* alleles.

684

Table 1. List of specimens tested on *Wolbachia* infection.

DNA ID	Species	Group			16S	*wsp*
		Complex	Subgroup			
AU402	*M. naxius* Vujić & Šašić, 2018	*luteomaculatus*	*bessarabicus*	*aureus* group	+	*
AU497	*M. erymanthius* Vujić, Ačanski & Šašić, 2018				+	A
AU812	*M. luteomaculatus* Vujić, Ačanski & Šašić, 2018				+	A
AU27	*M. euri* Vujić & Radenković, 2018				+	A
AU396	*M. peloponnesius* Vujić, Radenković, Ačanski & Šašić, 2018				+	A
AU504	*M. andriotes* Vujić, Radenković & Šašić, 2018				+	*
AU443	*M. sapphous* Vujić, Perez-Banon & Radenković, 2007	*sapphous*			+	AB
AU99	*M. saphous* sp. n. 1				+	A
AU141	*M. saphous* sp. n. 2				-	A
AU454	*M. bessarabicus* Paramonov, 1924	*bessarabicus*			+	AB
AU82	*M. bessarabicus* sp. n. 1				+	A
AU53	*M. ambiguus* Bradescu, 1986	*ambiguus*			+	A
AU474	*M. ambiguus* sp. n. 1				+	A
AU1435	*M. quercetorum* Marcos-García, Vujić & Mengual, 2007				+	A
AU1442	*M. legionensis* Marcos-García, Vujić & Mengual, 2007				+	A
AU1432	*M. nisi* Veselić, Vujić & Radenković 2017				+	-
AU321	*M. unicolor* Strobl, 1909	*unicolor*	*aureus*		+	AB
AU796	*M. unicolor* sp. n. 1				+	-
AU710	*M. aureus* Fabricius, 1805	*aureus*			+	AB
AU723	*M. aureus* sp. n. 1				+	A
AU701	*M. aureus* sp. n. 1				+	A
AU485	*M. aureus* sp. n. 2				-	A
AU360	*M. cinereus* (Fabricius, 1794)	*cinereus*	*cinereus*		+	**
AU1371	*M. aff. cinereus*				+	AB
AU530	*M. cinereus* sp. n. 1				+	A
AU1362	*M. cinereus* sp. n. 2				+	A
AU236	*M. cinereus* sp. n. 3				+	A
AU1443	*M. cinereus* sp. n. 4				+	A
AU517	*M. atratus* (Oldenberg, 1919)	*atratus*			+	A
AU151	*M. balkanicus* Šašić, Ačanski & Vujić, 2016				-	-
AU144	*M. virgatus* Vujić & Radenković, 2016				+	A
AU550	*M. virgatus*				+	A
AU874	*M. aerarius* Rondani, 1857				+	**
AU311	*M. minutus* Strobl, 1893	*chalybeus*	*chalybeus*		+	A
AU752	*M. chalybeus* Wiedemann, 1822				+	A
AU36	*M. dobrogensis* Bradescu, 1982	*dobrogensis*	*dobrogensis*		+	A
AU632	*M. dobrogensis*				+	A
AU413	*M. puniceus* Vujić, Radenković & Péres-Bañón, 2011				+	*
AU47	*M. dobrogensis* sp. n. 1				+	A

AU107	*M. caerulescens* Loew, 1869	*caerulescens*			+	A
AU176	*M. atricapillatus* Šašić, Ačanski & Vujić, 2018				+	A
AU742	*M. pumilus* Macquart, 1849				+	**
AU253	*M. pumilus*				+	**
AU115	*M. robustus* Veselić, Vujić & Radenković 2017				+	A
AU326	*M. unguicornis* Strobl, 1909				+	AB
AU272	*M. neofasciatus* Ståhls & Vujić, 2018	*geniculatus* group			+	nt
AU273	*M. neofasciatus*				+	nt
AU288	*M. neofasciatus*				+	nt
AU606	*M. albifrons* Meigen, 1822	*albifrons* group			-	nt
AU611	*M. albifrons*				-	nt
AU617	*M. albifrons*				-	nt
AU620	*M. constans* (Rossi, 1794)	*constans* group			+	nt
AU621	*M. constans*				+	nt
AU622	*M. constans*				+	nt
AU772	*M. segetum* (Fabricius, 1794)	*natans* group			+	nt
AU773	*M. segetum*				-	nt
AU775	*M. segetum*				+	nt
AU1146	*M. avidus* Rossi, 1790	*avidus* group			+	nt
AU1164	*M. avidus*				+	nt
KR1	*M. moenium* (Wiedemann in Meigen, 1822)				+	nt
KR2	*M. moenium*				+	nt
KR3	*M. moenium*				+	nt
N19	*M. nanus* Sack 1931	*nanus* group			+	nt
TS213	*M. nanus*				-	nt
TS219	*M. telmateia* Hurkmans, 1987				+	nt
TS221	*M. telmateia*				+	nt
TS222	*M. telmateia*				+	nt
NG15	*M. nigritarsis* Rondani, 1845	*nigritarsis* group			+	nt
NG16	*M. nigritarsis*				+	nt
NG17	*M. nigritarsis*				+	nt
AF55	*M. melanocerus* Bezzi, 1915	*desuturinus* group			+	nt
AF57	*M. melanocerus*				+	nt
AF58	*M. melanocerus*				-	nt
Y2367	*M. luteihumerus* Marcos-García, Vujić & Mengual, 2007				-	A
Y2368	*M. luteihumerus*				nt	A
Y2369	*M. luteihumerus*				nt	A
BULB	*Drimia maritima* (L.) Stearn; bulb	host plant of *M. luteihumerus*			-	A

A - *wsp* amplified using 136F/691R primer pair specific for supergroup A; B - *wsp* amplified using 81F/522R primer pair specific for supergroup B; AB - *wsp* amplified using 81F/522R universal primer pair; * - multiple products detected using electrophoresis; ** - poor sequence quality; nt - not tested.

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Table 2. The list of *wsp* alleles from *Merodon aureus* group host species including *Drimia maritima* bulb and *Merodon luteihumerus*.

Alleles	WSP profile*	Sequence ID (host species)
A1	311, 53, 145, 39, 18	AU497(*M. erymanthius*), AU812(*M. luteomaculatus*), AU27(*M. euri*), AU396(*M. peloponnesius*), AU82(*M. bessarabicus* sp. n. 1), AU99(*M. sapphous* sp. n. 1), AU141(*M. sapphous* sp. n. 2), AU53(*M. ambiguus*), AU723(*M. aureus* sp. n. 1), AU701(*M. aureus* sp. n. 1), AU236(*M. cinereus* sp. n. 3), AU144(*M. virgatus*), AU550(*M. virgatus*), AU752(*M. chalybeus*); AU36(*M. dobrogensis*), AU632(*M. dobrogensis*), AU47(*M. dobrogensis* sp. n. 1), AU107(*M. caerulescens*), AU176(*M. atricapillatus*); AU115(*M. robustus*), 16060(*Drimia maritima* plant bulb), Y2367(*M. luteihumerus*), Y2369(*M. luteihumerus*), Y2368(*M. luteihumerus*)
A2	731, 53, 145, 39, 18	AU311(*M. minutus*), AU1442(*M. legionensis*), AU1435(*M. quercetorum*)
A3	735, 28, 294, 39, 18	AU474(*M. ambiguus* sp. n. 1), AU443(*M. sapphous*)
A4	734, 261, 9, 271, 18	AU321(*M. unicolor*), AU710(*M. aureus*)
A5	(incomplete sequence)	AU454 (*M. bessarabicus*)
A6	733, 262, 115, 292, 62	AU326(*M. unguicornis*), AU485(*M. aureus* sp. n. 2)
A7	732, 263, 28, 31, 30	AU530(*M. cinereus* sp. n. 1), AU1362(*M. cinereus* sp. n. 2), AU517(*M. atratus*), AU1443(*M. cinereus* sp. n. 4), AU1371(*M. aff. cinereus*)

*WSP profile: *wsp*, HVR1, HVR2, HVR3, HVR4 IDs in *Wolbachia* MLST database (Baldo et al. 2006).

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Table 3. Uncorrected p distance values (%) between *wsp* alleles.

	A1	**A2**	**A3**	**A4**	**A5**	**A6**
A1						
A2	0.198					
A3	10.474	10.672				
A4	9.486	9.684	12.548			
A5	14.500	14.250	15.777	8.252		
A6	11.858	12.055	17.984	16.206	12.069	
A7	12.253	12.055	18.379	17.391	14.778	13.477

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